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Characterization and Partial Purification of a Novel Neuronotrophic Factor from Bovine Seminal Vesicle

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Abstract: Extracts from bovine seminal vesicles have been shown to contain high concentrations of nerve growth factor (NGF)-like biological activity and of the NGF protein with properties corresponding to that of NGF from other sources. We now demonstrate that a second neuronotrophic protein, termed seminal vesicle-derived neuronotrophic factor (SVNF), is present in seminal vesicle extracts (SVEs), which could not be distinguished from NGF on the basis of biological activity. SVNF has neuronotrophic activity on NGF target cells like embryonic chicken sensory and sympathetic neurons, sympathetic neurons, and chromaffin cells from neonatal rats, but it is inactive on embryonic chicken ciliary or neonatal rat nodose ganglion neurons. It also stimulates fiber outgrowth from rat pheochromocytoma (PC12) cells. In gel filtration chromatography on Biogel A 1.5 m, the activity is eluted with an apparent molecular weight of 40 kilodaltons, and by preparative isoelectric focusing, the isoelectric point was determined to be in the neutral range (6.8–7.8). The biological activity of SVNF, in contrast to that of NGF, is partially retained after preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and can be electrophoretically eluted with an apparent molecular weight of 16–20 kilodaltons. Electrophoretically purified SVNF is not inhibited by antisera to mouse NGF, but its activity is increased > 10-fold in the presence of very low concentrations of NGF. For partially purified SVNF, a specific activity of $2.9–5.8 \times 10^5$ biological units/mg of protein was determined in the presence of subthreshold NGF concentrations. Thus, two neuronotrophic proteins, SVNF and NGF, are present in high amounts in extracts from bovine seminal vesicles, exhibit very similar and cooperative biological activities, but differ in molecular weights, isoelectric point, and immunological properties. **Key Words:** Neuronotrophic factor—Nerve growth factor—Bovine seminal vesicle—Peripheral neurons. **Hofmann H.-D. and Unsicker K.** Characterization and partial purification of a novel neuronotrophic factor from bovine seminal vesicle. *J. Neurochem.* **48**, 1425–1433 (1987).

The regulation of neuronal development by epigenetic influences includes the action of soluble proteins, termed neuronotrophic factors (NTFs), that control growth and survival of neurons, particularly during phases of developmental cell death (Cowan, 1973; Jacobson, 1978; Varon and Adler, 1980; Hamburger and Oppenheim, 1982). Most of the evidence for this concept comes from studies of nerve growth factor (NGF) (Yankner and Shooter, 1982; Varon and Skaper, 1983). The biochemistry of NGF and its biological effects have been investigated extensively (Greene and Shooter, 1980; Calissano et al., 1984), because the protein could easily be purified from a number of tissues like the mouse submandibular gland, where, for unknown reasons, it is present in high concentrations (Cohen, 1960). NGF is still the only NTF for which the physiological relevance has been proven by the demonstration that injection of anti-NGF antibodies into perinatal animals results in degeneration of sympathetic and sensory ganglion cells (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). Numerous reports have described that various

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Abbreviations used: BDNF, brain-derived neuronotrophic factor; BU, biological unit; cCG8, 8-day-old chick embryo ciliary ganglia; cDRG8, cDRG10, and cDRG12, 8-, 10-, and 12-day-old chick embryo dorsal root ganglia, respectively; CMF, Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution; CNTF, ciliary neuronotrophic factor; cSG12, 12-day-old chick embryo lumbar sympathetic ganglia; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; kD, kilodaltons; NGF, nerve growth factor; NTF, neuronotrophic factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; rAM, neonatal rat adrenal medulla; rNG, neonatal rat nodose ganglia; rSCG, neonatal rat superior cervical ganglia; SDS, sodium dodecyl sulfate; SVE, seminal vesicle extract; SVNF, seminal vesicle-derived neuronotrophic factor.

tissue extracts and conditioned media exhibit survival-stimulating effects on cultured neuronal cells of peripheral and central origin (for review, see Varon and Adler, 1981; Barde et al., 1983; Berg, 1984) and contain neuronotropic agents distinguishable from NGF. Only two of these proteins have been purified so far: a ciliary NTF (CNTF) (Barbin et al., 1984) and a brain-derived neuronotrophic factor (BDNF) (Barde et al., 1982).

Both factors differ from NGF in their molecular and biological properties: BDNF is active on sensory neurons (Lindsay et al., 1985) but not on sympathetic neurons, whereas CNTF has survival-supporting activity for chicken ciliary neurons but not for sensory neurons from 8-day-old chicken embryos (Barbin et al., 1984). Studies on the physiological role of these NTFs, which would require the availability of antibodies against the active proteins, have not been reported so far. That purification of both proteins was achieved by using preparative polyacrylamide gel electrophoresis (PAGE) in the presence of the denaturing detergent sodium dodecyl sulfate (SDS) as a final purification step reflects the difficulties in obtaining pure preparations of neuronotrophic proteins, which are expected to be present at very low concentrations in the starting material.

In further investigations of the physiological role of NTFs and the underlying cellular and biochemical mechanisms, the search for neuronotrophic proteins with differing characteristics and their purification will remain an important aspect. We have previously reported (Hofmann and Unsicker, 1982) that extracts from bovine seminal vesicles contain high concentrations of NGF-like biological activity, and we could also demonstrate the presence of large amounts of the NGF protein. Thus, the seminal vesicle seems to be the main source of NGF activity found in bovine seminal plasma (Harper and Thoenen, 1980). The biochemical and biological properties of bovine NGF isolated from seminal plasma were virtually identical to that of mouse submandibular gland NGF (Harper et al., 1982, 1983). We now show that part of the biological activity found in extracts from bovine seminal vesicles is due to a protein that is different from NGF in its biochemical and immunological properties but surprisingly similar to NGF with regard to its biological effects on cultured neurons.

MATERIALS AND METHODS

NGF was purified from male mouse submandibular glands by the method of Bocchini and Angeletti (1969) as modified by Suda et al. (1978) using an additional gel filtration step on Sephadex G 75. Antisera to mouse NGF were raised in rabbits and characterized as described previously (Hofmann and Unsicker, 1982). Unfractionated antisera or IgG fractions after ammonium sulfate precipitation (45% saturation) were used.

Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (CMF) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Eggenstein, F.R.G.). Fetal calf serum (FCS), horse serum, RPMI 1640 medium, trypsin, streptomycin, and penicillin were from Biochrom KG (Berlin, F.R.G.). Poly-L-ornithine (mol wt, 30,000), bovine serum albumin, and soybean trypsin inhibitor were from Sigma Chemie (Deisenhofen, F.R.G.). Cytochrome c, chymotrypsinogen A, ovalbumin, acrylamide, bisacrylamide, Servalytes (pH 4–9 and 9–11), and phosphate-buffered saline (PBS) were all from SERVA (Heidelberg, F.R.G.). Biogel A 1.5 m was from BioRad Labs (München, F.R.G.), and Sephadex G 75 and G 200 was from Pharmacia (Uppsala, Sweden). Goat anti-rabbit IgG antiserum and rabbit peroxidase antiperoxidase complex were purchased from Bayer Diagnostik (München).

CMF was supplemented with $NaHCO_3$ to 44 m*M* and penicillin/streptomycin (100 units/ml each). Culture medium was DMEM containing 10% heat-inactivated FCS, 2 m*M* L-glutamine, 44 m*M* $NaHCO_3$, and 100 units/ml of penicillin and streptomycin.

Fertilized chick eggs were obtained from a local aviary and incubated in a humidified egg chamber at 37.8°C. Pregnant Hannover-Wistar rats were purchased from Ivanovas (Kisslegg, F.R.G.).

Preparation of seminal vesicle extract

Bovine seminal vesicles were dissected from freshly slaughtered adult bulls, transported on ice, and thoroughly freed of connective tissue, and the glandular tissue was immediately homogenized in 2 volumes (wt/vol) of ice-cold 2 m*M* sodium phosphate buffer, pH 7.2, using a Waring blender. After centrifugation at 15,000 *g* for 20 min and 100,000 *g* for 60 min, the supernatant was successively filtered through membrane filters with pore sizes of 3 and 0.45 μm. The resulting crude seminal vesicle extract (SVE) could be stored at −20°C for 6 months without loss of biological activity. For further processing by gel filtration chromatography and preparative isoelectric focusing, SVE was lyophilized, dissolved fivefold concentrated in water, dialyzed against water for 24 h, and centrifuged at 15,000 *g* for 30 min. The clear supernatant was carefully separated from a top layer of lipid material and directly applied to the gel filtration column or to the preparative focusing gel, which was prepared and run as previously described (Hofmann and Unsicker, 1982).

SDS-PAGE and immunoblotting

For analytical and preparative SDS-PAGE, the buffer system of Laemmli (1970) was used. Linear polyacrylamide gradient slab gels (7.5–20%, 1.5 mm thick) were prepared and electrophoresed as described by Anderson and Peterson (1981). Analytical gels were stained with Coomassie Blue (0.25% Coomassie Blue in 9% acetic acid/45% methanol) for 1 h and destained in 5% acetic acid/7.5% methanol or used for electrophoretic transfer of separated proteins to nitrocellulose, essentially by the procedure of Burnette (1981). Proteins binding anti-NGF antibodies were identified by the peroxidase antiperoxidase technique (Hofmann and Unsicker, 1982) using anti-NGF antisera at a 100-fold dilution. Standard proteins used were bovine serum albumin [68 kilodaltons (kD)], ovalbumin (45 kD), carbonic anhydrase (29 kD), chymotrypsinogen A (25 kD), β-lactoglobulin (18.4 kD), and cytochrome c (12.8 kD).

Stacking gels for preparative SDS-PAGE were formed with the use of a comb having a broad center tooth (11 cm) and two small outer teeth (0.5 cm). Samples were not dena-

tured before loading and mercaptoethanol was omitted from the sample buffer, because no biological activity was recovered in the presence of reducing agents. The seminal vesicle-derived NTF (SVNF) pool after gel filtration chromatography was concentrated 12-fold by ultrafiltration through Amicon PM10 membranes before application to the gel. The outer wells were loaded with 20 μg of cytochrome c, the position of which was visible after separation when gels were cut into 2-mm strips, starting from the position of cytochrome c (M_r 12,800) up to a molecular weight range of 45,000. On both sides, a small part of the center lane was left uncut. This procedure allowed the localization of the position of each strip after the uncut parts of the gel were stained. The gel strips were cut into small pieces, and proteins were electrophoretically eluted overnight in tubes (1 cm in diameter) through a 6% polyacrylamide disc gel containing 6 *M* urea as described by Hanaoka et al. (1979). The proteins, freed of SDS, were collected from the bottom part of the tube in ~1 ml of buffer and were directly assayed for biological activity or stored at −80°C.

Bioassays

All cell types were cultured in 96-well microtiter plates (Falcon) precoated with polyornithine and rat C6 glioma cell conditioned medium diluted fourfold with DMEM. Pretreatment of the culture plates and preparation of the conditioned medium have been described previously (Barbin et al., 1984; Unsicker et al., 1984). PC12 cells were cultured on polyornithine alone. Samples to be tested were diluted serially twofold with DMEM containing 10% FCS, and 50 μl were added per well. Each sample was assayed in triplicate. The biological activity of SVNF and NGF was routinely determined as the ability to support survival of neurons dissociated from 8-day-old embryonic chick dorsal root ganglia (cDRG8) during a 24-h culture period. Dissected ganglia were collected in CMF, treated with 0.11% trypsin in CMF for 15 min at 37°C, and washed twice with DMEM containing 10% FCS. Cells were mechanically dissociated by trituration through a flame-narrowed pasteur pipette (yield, 20,000–25,000 cells/ganglion). Two thousand cells (950–1,100 neurons) were seeded per well in 50 μl of DMEM containing 10% FCS and cultured at 37°C in a 5% CO_2/95% air atmosphere for 24 h. Cells were fixed with 2.5% glutaraldehyde in PBS, and the number of phase-bright surviving neurons per well was determined by counting one diametrical strip, representing 10% of the total area of the well, under phase-contrast optics. The number of neurons surviving was 50–100 in control cultures and 300–500 in the presence of saturating concentrations of trophic factors. The dilution of NTF-containing samples that supported half-maximal survival of neurons was defined to contain 1 biological unit (BU)/ml.

Dissociation procedures and culture conditions for other peripheral neurons and rat chromaffin cells, which were also tested for their responsiveness to SVNF and NGF, were very similar to that described for cDRG with the following modifications: Chick embryo sympathetic ganglia from 12-day-old embryos (cSG12) and 8-day-old chick embryo ciliary ganglia (cCG8) were treated with 0.125 and 0.08% trypsin, respectively, and cSG were seeded at a cell density of 1,000 cells/well. Neonatal rat superior cervical ganglia (rSCG) and neonatal rat nodose ganglia (rNG) were dissected and dissociated using the same enzymatic procedure as the one used for mouse dorsal root ganglia (Skaper et al., 1980). Chromaffin cells were dissociated as described previously (Unsicker et al., 1985) and cultured in DMEM supplemented with 20% FCS for 4 days.

The rat pheochromocytoma (PC12) cell line was maintained in culture as described by Greene and Tischler (1976). The ability of SVNF and NGF to stimulate neurite formation in these cells was tested. PC12 cells were seeded at a density of 1,000 cells/well using RPMI 1640 medium supplemented with 10% horse serum, 5% FCS, 2 m*M* glutamine, and 100 units/ml each of penicillin and streptomycin as culture medium. The cells were cultured for 72 h and fixed, and the percentage of cells having processes longer than one cell diameter was determined by counting 100 cells/well.

Isolation of bovine NGF and protein content determination

Bovine NGF was purified from the NGF pool after gel filtration chromatography (see Results) by affinity chromatography on a column with anti-mouse NGF immunoglobulins coupled to Sepharose 4B. IgGs were precipitated from rabbit anti-NGF antisera with ammonium sulfate (45% saturation) and coupled to cyanogen bromide-activated Sepharose 4B. Chromatography was performed as described by Stoeckel et al. (1976). Bovine NGF eluted at pH 2 from the column with 50 m*M* glycine HCl was dialyzed against PBS before use. Protein concentrations were measured according to the method of Lowry et al. (1951).

RESULTS

Separation of SVNF and NGF by gel filtration chromatography and preparative isoelectric focusing

When concentrated and dialyzed SVE (see Materials and Methods) is run on a Biogel A 1.5 m gel filtration column and the fractions are directly assayed for their survival-stimulating activity on cDRG8 neurons, the biological activity is found in two peaks (Fig. 1). The elution volumes of the fractions with maximal activity correspond to apparent molecular weights of 37–40 (SVNF) and 26–30 kD (NGF), respectively, with the latter value being in good agreement with the molecular weight of mouse β-NGF (26.5 kD). In Fig. 1, both peaks contain ~50% of total activity recovered from the column. However, the percentage of activity in the SVNF peak varied considerably for different SVE preparations, usually between 30 and 50%, and in some cases it was even lower. It is not yet clear whether this variability is due to experimental conditions or to variations in the biological material.

Using preparative isoelectric focusing for separating SVE proteins again results in two activity peaks, one at a basic pI range of 8.9–9.3, which is similar to that of mouse NGF, and a second one focusing at almost neutral pH (Fig. 2A). Again, the amount of activity that is recovered in the second peak is not constant for different SVE preparations. This finding could indicate that basic NGF partially bound to acidic proteins gives rise to an additional activity peak. Therefore, the active fractions at pH 6.8–7.8 were pooled from three runs, concentrated by ultrafiltration, and refocused under identical conditions. During this second run,

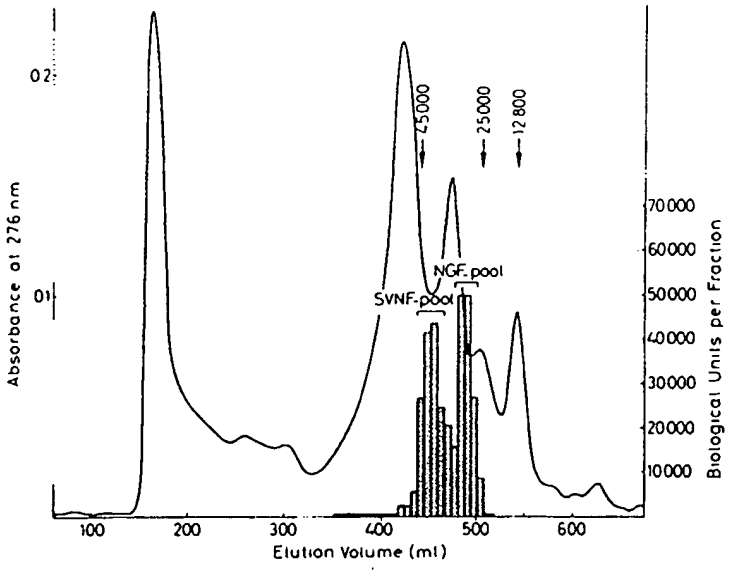

FIG. 1. Gel filtration chromatography of SVE on Biogel A 1.5 m. Four milliliters of lyophilized, concentrated, and dialyzed SVE (see Materials and Methods) corresponding to 2.4×10^6 BU was applied to a 2.6- × 120-cm Biogel A 1.5 m column equilibrated with PBS, and the column was eluted at a flow rate of 22 ml/h with the same buffer. Fifteen-minute fractions were collected, and each fraction was tested for survival supporting activity on cDRG8 neurons (histogram). Fractions that were combined to give the SVNF and NGF pools are indicated. Positions of marker proteins from parallel runs (cytochrome c, M_r 12,800; ovalbumin, M_r 45,000; bovine serum albumin, M_r 68,000) are shown by arrows.

no NGF dissociates to focus at pH 9.0–9.3, as would be expected if the peak at neutral pH was caused by NGF molecules nonspecifically bound to other proteins (Fig. 2B).

Analysis of the active fractions from gel filtration chromatography and from preparative isoelectric focusing by SDS-PAGE and immunoblotting with antibodies to mouse NGF demonstrates that two different active molecules are present in SVE, both of which stimulate the survival of cDRG8 neurons in culture (Fig. 3). Antibodies raised against 2.5S NGF from mouse submandibular glands clearly identify bovine NGF molecules present in SVE, in the NGF pool after gel filtration chromatography, and in the active fraction with pI 9.1 from preparative isoelectric focusing (Fig. 3A, lanes 4 and 6, and B, lane 4, respectively), although because of incomplete immunological cross-reactivity, staining of bovine NGF is less intense than that of mouse 2.5S NGF (M_r 12,000), which was run for comparison (Fig. 3A, lane 7, and B, lane 5). In the presence of SDS, the molecular weight of bovine NGF is almost identical to that reported for mouse β-NGF monomer (13 kD). Corresponding SVNF fractions having the same biological activity but exhibiting non-NGF-like biochemical properties contain no or almost undetectable amounts of protein that binds anti-NGF antibodies on the immunoblot (Fig. 3A, lane 5, and B, lane 3). In the case of the SVNF pool, a very faint staining is possibly caused by overlapping of the two activity peaks in gel filtration chromatography.

Isolation of SVNF by preparative SDS-PAGE

Another criterion that biochemically distinguishes SVNF and NGF is their behavior in SDS-PAGE. It was not possible, in our hands, to regain biologically

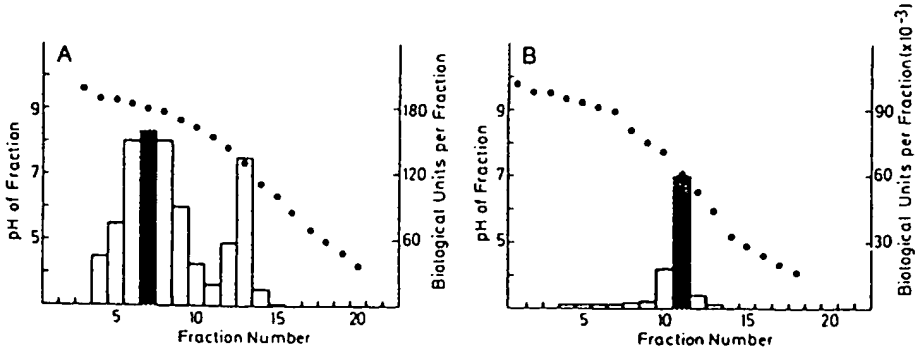

FIG. 2. Preparative isoelectric focusing of SVE and refocusing of pooled "neutral" fractions. A: Three milliliters (1.8×10^6 BU) of concentrated SVE was loaded on the Sephadex G 200 gel containing 3.75% Servalyte 4–9 and 2.5% Servalyte 9–11. After focusing, 30 fractions were eluted in 3 ml of water each, and the pH (●) and biological activity (histogram) were determined. B: Fractions at pH 6.8–7.8 from three runs as described in A were pooled, and 3 ml of this pool (150,000 BU) was refocused under identical conditions. Black columns in A and B indicate the fractions that were analyzed by SDS-PAGE and immunoblotting (see Fig. 3).

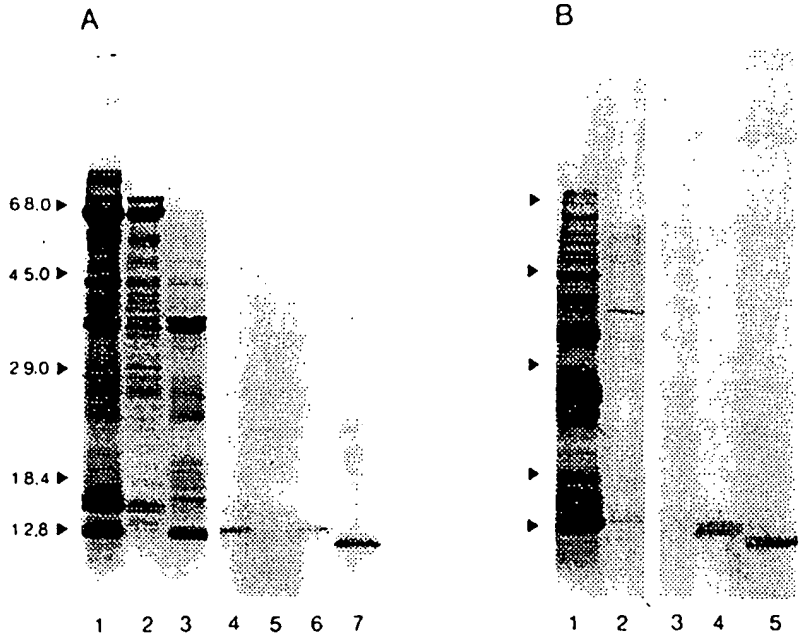

FIG. 3. SDS-PAGE and immunoblotting of SVE fractions. A: Lanes 1 and 4, SVE (1,000 BU); lanes 2 and 5, SVNF pool (1,500 BU); lanes 3 and 6, NGF pool (1,500 BU); and lane 7, mouse 2.5S NGF (500 BU). B: Precipitated and redissolved proteins. Fractions from gel filtration chromatography and preparative isoelectric focusing were analyzed on SDS-polyacrylamide gradient gels (7.5–20%). Proteins were either stained with Coomassie Blue (A, lanes 1–3; B, lanes 1 and 2) or electrophoretically transferred to nitrocelulose paper (A, lanes 4–7; B, lanes 3–5), and protein bands binding anti-NGF antibodies were visualized by the peroxidase antiperoxidase method. In B, from appropriate volumes of isoelectric focusing fractions, proteins were precipitated in 0.3 *M* trichloroacetic acid, centrifuged, and redissolved in sample buffer. Lanes 1 and 3, SVNF fraction with neutral pH (10,000 BU) (see Fig. 2B); lanes 2 and 4, basic NGF fraction (10,000 BU) (see Fig. 2A); and lane 5, mouse NGF (500 BU). Arrowheads indicate the positions of standard proteins with molecular weights in kD.

active NGF after SDS-PAGE by electrophoretically eluting the separated proteins from gel slices as described in Materials and Methods, whether purified mouse NGF or the NGF pool after gel filtration chromatography was used as the sample. After fractionation of SVNF pool proteins on preparative SDS-polyacrylamide gels, however, biological activity is partially retained and localized at a position that is clearly different from that of the NGF monomer (Fig. 4). The activity peak does not correlate with one of the Coomassie Blue-stained bands, and it is less sharp than would be expected from protein staining, because it was not possible to cut the unstained preparative gels exactly parallel to the protein bands. Maximal activity was reproducibly measured in a molecular weight range of 16–20 kD (Fig. 4).

On average, 5% of the activity applied to the preparative gel was recovered in the active fractions. This low yield must be partially ascribed to incomplete restoration of activity after denaturation by SDS, but, as will be discussed below (see also Fig. 6), complete separation of NGF and SVNF could be the main reason for the dramatic decrease in biological activity after SDS-PAGE.

Characterization of the biological activity of SVNF

In accordance with results obtained by immunoblot analysis (Fig. 3), antibodies to mouse NGF do not interfere with survival-stimulating activity of electrophoretically purified SVNF in the cDRG8 bioassay. Even at concentrations as low as 1 BU/ml, corresponding to half-maximal survival-supporting activity, SVNF is not inhibited up to an antiserum concentration of 10 μl/ml. The effect of 5 BU/ml of bovine NGF is completely blocked by addition of 3 μl of antiserum/ml (Fig. 5). The titer of the antiserum for mouse NGF is even 10 times higher (data not shown).

The results described so far clearly demonstrate that the active protein present after electrophoretic purification of SVNF is different from the well-characterized NGF molecule. Therefore, we used SVNF at this

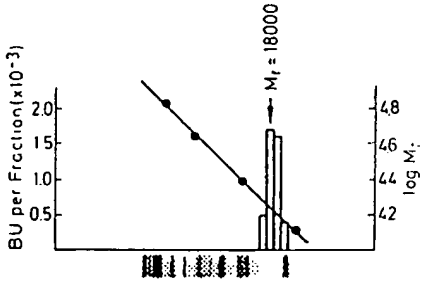

FIG. 4. Preparative SDS-PAGE of the SVNF pool from gel filtration chromatography. Two hundred microliters of a 12-fold concentrated SVNF pool was mixed with 3× sample buffer (without mercaptoethanol) and run on a 7.5–20% gradient resolving gel and 5% stacking gel. The gel was cut into 2-mm strips, and the separated proteins were eluted electrophoretically through gels containing 6% polyacrylamide gel (see Materials and Methods). Eluted proteins were directly assayed for biological activity (histogram). A corresponding Coomassie Blue-stained portion of the gel is shown below. The gel was calibrated by correlating the protein bands with those of standard proteins in a parallel analytical gel run under identical conditions (●). Cytochrome *c* (12.6 kD), chymotrypsinogen A (25 kD), ovalbumin (45 kD), and bovine serum albumin (68 kD) were used as standard proteins.

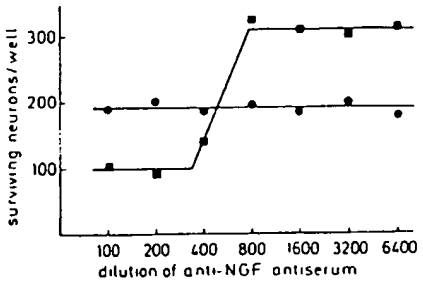

FIG. 5. Effects of antibodies to mouse NGF on biological activities of SVNF and bovine NGF. Serial twofold dilutions of anti-mouse NGF antiserum were incubated with constant amounts of electrophoretically purified 1 BU/ml of SVNF (●) or 5 BU/ml of bovine NGF (■) for 1 h at 37°C, and the bioassay was performed as described in Materials and Methods. Curves were fitted by eye.

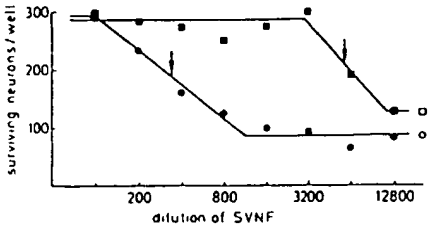

FIG. 6. Titration of the survival-supporting activity of SVNF in the presence and absence of NGF. Serial twofold dilutions of electrophoretically purified SVNF were tested for their ability to support survival of cDRG8 neurons in culture under standard conditions (●) or with purified mouse 2.5S NGF (0.1 BU/ml) added (■). Open symbols indicate the corresponding control values in the absence of SVNF. Curves were fitted by eye. Arrows indicate the dilution at half maximal activity (350-fold under standard conditions; 5,600-fold in the presence of NGF).

stage of purification to compare its target cell specificities with that of purified mouse NGF. As summarized in Table 1, SVNF supports survival of all NGF-responsive cell types tested [cDRG8, cDRG10, and cDRG12; cSG12; rSCG; and neonatal rat adrenal medulla (rAM)] and has no effect on cCG8 and rNG neurons, which are known to be NGF independent in culture. On PC12 cells, both factors are able to stimulate process formation.

Using saturating NTF concentrations, as determined in the cDRG8 assay system, significant quantitative differences between SVNF and NGF with regard to the number of supported cells are only found for rSCG and for PC12 cells regarding the proportion of process-bearing cells. For all other cell types, both NTFs were equally effective, and no additive effects were observed when both proteins were present, a result suggesting that NGF and SVNF address the same subpopulation of neurons. That these very similar effects of the two NTFs, however, are achieved by different mechanisms of action is indicated by the findings shown in Fig. 6. In this experiment, SVNF activity on cDRG8 neurons was titrated under standard conditions or in the presence of added NGF (0.1 BU/ml). This very low NGF concentration does not, by itself, significantly increase the number of surviving neurons but leads to a >10-fold increase of the SVNF activity in terms of BU/ml.

Cooperative action of SVNF and NGF has to be taken into consideration when yields of activity and specific activities are calculated in the purification procedure of SVNF from SVE that contains high activities of both factors. This is particularly relevant for the electrophoretical purification step, which com-

TABLE 1. *Comparison of the biological activities of electrophoretically purified SVNF and mouse NGF*

Cell type	No factor added	SVNF (20 BU/ml)	NGF (20 BU/ml)	SVNF + NGF (20 BU/ml each)
cDRG8	80 ± 20	497 ± 60	378 ± 110	550 ± 100
cDRG10	180 ± 50	560 ± 80	560 ± 100	620 ± 60
cDRG12	286 ± 35	442 ± 50	552 ± 75	640 ± 62
cSG12	170 ± 36	370 ± 40	320 ± 50	ND
cCG8	30 ± 12	30 ± 20	50 ± 13	50 ± 13
rNG	145 ± 35	105 ± 30	125 ± 30	ND
rSCG	300 ± 80	500 ± 100	930 ± 120	ND
rAM	60 ± 30	140 ± 20	160 ± 50	ND
PC12	0	28 ± 3	47 ± 7	54 ± 4

Data are surviving neurons per well, except for PC12 cells, which are given as percentage of neurite-bearing cells. Results are mean ± SEM values from five or more separate experiments. ND, not determined.

TABLE 2. *Biological activities of SVE fractions*

	Protein concentration (mg/ml)	Biological activity (BU/ml, $\times 10^{-3}$)	Specific activity (BU/mg of protein, $\times 10^{-3}$)
SVE	17.2	150.0	8.7
SVNF pool (Biogel A 1.5 m)	0.65	25.0	38.5
NGF pool (Biogel A 1.5 m)	0.68	28.0	41.0
SVNF (SDS-PAGE)			
Without added NGF	0.45	1.6	35.5
With NGF	—	13.0–26.0	289.0–578.0

Values for SVE are averaged from three preparations. For the SVNF pool and NGF pool, data were obtained by pooling the fractions of six Biogel A 1.5 m columns from three different SVE preparations. For electrophoretically purified SVNF, the biological activity was determined under standard conditions and in the presence of NGF (0.1 BU/ml), as shown in Fig. 6. Values are the means from three preparative gels, using the fraction with the highest activity in each case.

TABLE 3. *Comparison of the properties of SVNF and NGF*

	SVNF	NGF
Apparent molecular weight		
on Biogel A 1.5 m	~40 kD	26 kD[a]
in SDS-PAGE	16-20 kD	13 kD
Isoelectric point	6.8-7.8	9.0-9.3[a]
Trophic activity for	cDRG8-12, cSG12, rSCG, rAM, PC12	
No activity for	cCG8, rNG	
Recognition by anti-NGF antibodies	No	Yes
Activity enhanced by NGF	Yes	—

Data for NGF are derived from results described in this article unless stated otherwise.
[a] Determined separately using purified mouse β-NGF.

pletely removes active NGF from SVNF-containing fractions. For that reason, yields and purification factors are not presented in Table 2, but activities of electrophoretically purified SVNF are given for the two different assay conditions described in Fig. 6. In different experiments, the factor by which SVNF activity was enhanced in the presence of NGF varied between 8 and 16, corresponding to a specific activity of $2.9–5.8 \times 10^5$ BU/mg of protein. This value is in the range of specific activities described for purified CNTF (Barbin et al., 1984) and BDNF (Barde et al., 1982), although SVNF is still not purified to homogeneity after preparative SDS-PAGE.

Table 3 shows a comparative summary of the characteristics of SVNF and NGF studied in this article and demonstrates that the two NTFs differ in all biochemical properties determined and in their susceptibility to anti-NGF antibodies but are very similar with regard to their target cell spectrum.

DISCUSSION

NGF has become the prototype NTF since it was purified from an extremely rich source, the submandibular glands of male mice (Cohen, 1960). For that reason, approaches to identify and isolate new NTFs normally start with the establishment of an assay system that allows discrimination between NGF and other active molecules either by using neurons that are not responsive to NGF or by the use of anti-NGF antibodies (Varon and Adler, 1981). In the case of SVNF, both methods were not applicable because (a) high concentrations of NGF are present in the starting material; (b) as was recognized later, SVNF affects the same spectrum of neuronal cell types as NGF; and (c) NGF interferes with the quantitative determination of SVNF activity.

Using analytical SDS-PAGE and an immunoblot technique, it was possible, however, to demonstrate that SVE contains, in addition to classical NGF, another neuronotrophic protein with an apparent molecular weight of ~40 kD and an isoelectric point in the neutral pH range (6.8–7.8), determined by gel filtration chromatography and preparative isoelectric focusing, respectively. In contrast to NGF, the new factor can be further purified by SDS-PAGE in the absence of reducing agents with partial retention of biological activity. Comparison of molecular weights determined by gel filtration chromatography (40 kD) and SDS-PAGE (16–20 kD) suggests that SVNF, like NGF, exists as a dimer under native conditions.

Together with the finding that survival-supporting activity of electrophoretically purified SVNF is not reduced in the presence of anti-NGF antibodies, the biochemical data provide strong evidence for the expression of two different polypeptides with very similar neuronotrophic activity in bovine seminal vesicles. Our results, however, do not exclude the interpretation that SVNF could be a precursor molecule of NGF or could be produced from such a precursor by different processing steps. Such an interpretation would be in agreement with results from biochemical studies on the protein synthesis of mouse submandibular gland β-NGF (Berger and Shooter, 1977) and with cDNA sequencing data (Ulrich et al., 1983). In any case, the resulting polypeptide, in contrast to mouse NGF and other mammalian NGFs (Harper and Thoenen, 1980), does not react with antibodies raised against mouse NGF and, as concluded from the synergistic action of both NTFs (Fig. 6), acts via a biochemical mechanism different from that of NGF. This conclusion is further supported by [^{125}I]NGF binding studies on PC12 cells, which did not indicate any influence of SVNF on NGF receptor binding (data not shown).

In view of the distinctly different biochemical and immunological properties, it was intriguing to find no differences in target cell specificity between NGF and SVNF. The cell types tested so far, however, necessarily represent an incomplete selection of putative target cells, and it may well be that our results only reflect a high degree of overlapping in cell specificity. Results reported for CNTF, the only neuronotrophic protein besides NGF purified from a peripheral neuronal target tissue, suggest that broad overlapping target cell spectra are not exceptional for NTFs (Barbin et al., 1984; Unsicker et al., 1985). Quantitatively, we found differences in the capability of SVNF and NGF to stimulate survival of neonatal rat sympathetic neurons and fiber outgrowth on PC12 cells. This aspect, however, was not investigated in detail and may be a consequence of the fact that PC12 cells and chromaffin cells need higher NTF doses for maximal response compared with sensory and sympathetic neurons (Greene, 1978; Unsicker et al., 1985). Concern-

ing the characteristics of SVNF activity in comparison to those of NGF, it was a particularly interesting observation that in the presence of subthreshold concentrations of added NGF, the biological activity of SVNF determined in the cDRG8 assay system is enhanced > 10-fold in terms of BU/ml. A similar finding was reported recently for the simultaneous action of CNTF and NGF (Unsicker et al., 1985). These cooperative effects suggest that NTFs may act via different but interacting cellular mechanisms.

The significance of two factors with similar neuronotrophic activities being present in one organ at very high concentrations, in terms of neuronotrophic activity, is not clear. It is of interest that the coexistence of NGF and a protein termed neurite-inducing factor in extracts from mouse submandibular glands was described recently (Wagner, 1986). Neurite-inducing factor, like NGF, stimulates neurite formation and ornithine decarboxylase activity in PC12 cells. It was partially purified by SDS-PAGE, has an apparent molecular weight of 20 kD in the presence of SDS, and was shown to be less basic than NGF, thus having striking similarities to the protein described here.

In summary, we were able to identify a novel NTF that coresides with high concentrations of NGF in extracts from bovine seminal vesicles. The protein could be distinguished from NGF by biochemical and immunological criteria. It was partially purified by gel filtration chromatography and preparative SDS-PAGE, and its biological activity was shown to resemble that of NGF.

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